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# **Deletions of 20p12 in Alagille Syndrome:** Frequency and Molecular Characterization

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**Alagille syndrome is an autosomal dominant disorder comprising cholestasis (associated with intrahepatic bile duct paucity), characteristic facial appearance, and cardiac, ocular and skeletal defects. Multiple patients have been reported with deletions or translocation involving 20p11.23-p12, providing evidence for the localization of the disease gene to this region. Fifty-six Alagille syndrome patients have been studied by cytogenetic and/or molecular analysis to determine the frequency of detectable abnormalities of 20p12. Two of fifty-six patients studied by cytogenetic analysis had abnormalities: an interstitial deletion in one patient and a translocation in another. Of forty-five patients studied by molecular analysis, three were found to have deletions of 20p, including the two patients identified with cytogenetic abnormalities. Molecular and molecular cytogenetic (FISH) analysis of the translocation (46,XX,t(2;20)(q21.3p12)) demonstrated a deletion at the translocation breakpoint. The deletions identified in the three patients are overlapping, contributing to the delineation of an Alagille syndrome critical region within 20p12. This region lies between markers D20S41 and D20S162. The frequency of detectable cytogenetic abnormalities of 20p12 in this group of Alagille patients is 2/56 (3.6%), and the frequency of molecular deletions is 3/45 (6.7%). This is considerably lower than the frequency of deletions observed in contiguous gene deletion syndromes suggesting that Alagille syndrome may be caused by the alteration of a single gene. Am. J. Med. Genet. 70:80-86, 1997.** © 1997 Wiley-Liss, Inc.

**KEY WORDS: 20p deletions; Alagille syndrome; bile duct paucity and cardiac disease**

## INTRODUCTION

Alagille syndrome (syndromic bile duct paucity, arteriohepatic dysplasia, McKusick number 118540) is one of the major forms of chronic liver disease in childhood and has a minimal estimated frequency of 1/100,000 live births [Danks et al., 1977]. It is characterized by intrahepatic bile duct paucity on biopsy and five main clinical anomalies (cholestasis, cardiac disease, skeletal abnormalities, ocular abnormalities, and a recognizable facial phenotype [Alagille et al., 1987]). Cardiac anomalies most commonly involve the peripheral and main pulmonary arteries and valves [Greenwood et al., 1976]. The most common skeletal anomalies are "butterfly" or hemivertebrae, resulting from clefting abnormalities of the vertebral bodies [Rosenfield et al., 1980]. Ocular lesions include anterior chamber defects, most commonly posterior embryotoxon (a benign defect), and retinal pigmentary abnormalities [Puklin et al., 1981]. Patients have prominent forehead, deep-set eyes, hypertelorism, long straight nose with flattened tip, short philtrum, flat midface, and triangular chin. Renal and neurodevelopmental abnormalities occur less frequently [Alagille et al., 1987]. Fifteen percent of patients will require a liver transplant [Piccoli and Witzleben, 1991]. Seven to ten percent of patients have severe congenital heart disease,

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most often a severe form of tetralogy of Fallot [Piccoli and Witzleben, 1991].

Alagille syndrome is an autosomal dominant trait, although expressivity is highly variable [LaBrecque et al., 1982; Shulman et al., 1984]. Penetrance was estimated at 94% if only one of the five main clinical anomalies is observed in an affected relative [Dhorne-Pollet et al., 1994]. The frequency of new mutations was estimated as 15% in one study [Dhorne-Pollet et al., 1994] and 50% in a second study [Elmslie et al., 1995]. The variable expressivity makes diagnosis of carriers difficult, and without definitive clinical or genetic markers, counseling for recurrence risks is likely to be problematic.

Multiple patients have been identified with cytogenetic abnormalities of 20p11-12, suggesting that the disease gene lies in this region [Anad et al. 1990; Byrne et al., 1986; Legius et al., 1990; Schnittger et al., 1989; Teebi et al., 1992; Spinner et al., 1994 ; Zhang et al., 1990] with the common region of overlap of the deletions mapping to bands 20p11.23-12. Previously we have identified a submicroscopic deletion of 20p12 in 1/24 patients screened [Rand et al., 1995].

The purpose of this study was to determine the frequency of cytogenetic and molecular deletions in a group of individuals, to compare the deletions in three patients that we have identified, and to compare the clinical characteristics in deleted and non-deleted patients.

## PATIENTS AND METHODS

Fifty-six patients were referred with the diagnosis of Alagille syndrome. Informed consent was obtained from the parents of all children for participation in the study. Criteria for diagnosis included liver biopsy findings consistent with Alagille syndrome and at least three of the five primary clinical criteria: cholestasis, characteristic face, posterior embryotoxon, "butterfly" vertebrae, and cardiac findings [Alagille et al., 1987]. If there was a family history of Alagille syndrome (as judged by the criteria listed above) an individual was considered affected if two of the criteria were present [Dhorne-Pollet et al., 1994].

Metaphase chromosome analysis was performed on all 56 patients at the 500–850 band level of resolution using standard techniques. Analysis was carried out on cells from peripheral blood or from lymphoblastoid cell lines, which were established whenever possible.

Molecular deletions were assayed by analysis of polymorphic loci from 20p. Microsatellite markers for 20p12 were purchased from Research Genetics, Inc. (Huntsville, AL) with the exception of AFM164TG5, which was identified through the Whitehead Institute for Biomedical Research Database and primers were synthesized. Analysis was carried out on the index-case and both parents (where available) using the PCR method as described previously [Rand et al., 1995]. Molecular analysis was carried out in 45 patients. DNA for the PCR analysis was not available on 11 of the patients.

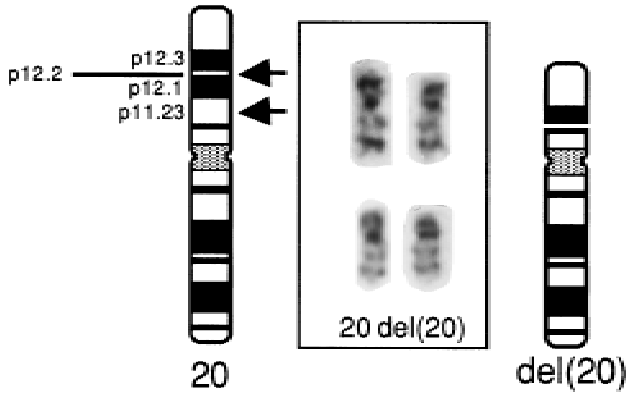


Fig. 1. Partial G-banded karyotypes of chromosome 20 from patient 1 showing the del(20)(p11.23p12). An ideogram demonstrates the locations of the breakpoints.

For fluorescence in situ hybridization (FISH), clone pRI2.21 containing the locus D20S5 (ATCC, Rockville, MD) was labeled with biotin or digoxigenin by nick translation using commercially available kits (Gibco-BRL, Gaithersburg, MD). Labeled DNA was combined with Cot-1 and herring sperm DNA and in some experiments an alpha satellite probe for chromosomes 2 and/or 20 was used. Hybridization and washes were done using standard conditions and DAPI or propidium iodide was used as a counter stain [Lichter and Cremer, 1992].

## RESULTS

### Cytogenetics and Case Report

Two of the 56 patients studied by cytogenetic analysis had abnormalities involving 20p12 (3.6%). Patient 1 had a 46,XX, del(20)(p11.23p12) karyotype (Fig. 1). She was the second child born to healthy, non-consanguineous parents at 38 weeks after an uncomplicated pregnancy. A murmur was noted at birth and subsequent echocardiogram demonstrated tetralogy of Fallot with pulmonary stenosis at multiple levels. She failed to thrive and was admitted for a systemic to pulmonary shunt at age 3 weeks. Examination at that time showed a symmetrically growth-retarded, jaundiced child with a large anterior fontanel, short palpebral fissures, bulbous nasal tip, notched alae nasi, and bi-

TABLE I. Deletion Analysis of Selected Chromosome 20p Loci in 3 Alagille Syndrome Patients With Deletions of 20p*

| Centromere | D20S61 | D20S41 | **D20S186** | **D20S189** | **D20S27** | **D20S188** | **AFM164 TG5** | D20S162 | D20S5 | D20S175 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. del(20) | h | h | **u** | **d** | **u** | **u** | **u** | d | nt | d |
| 2. t(2;20) | h | h | **u** | **u** | **u** | **u** | **d** | d | d | h |
| 3. 46,XX | d | d | **d** | **u** | **u** | **d** | **u** | h | nt | h |

*d, deleted; h, heterozygous (two alleles present); u, uninformative; nt, not tested. Markers in bold cannot be excluded from the minimal commonly deleted region.

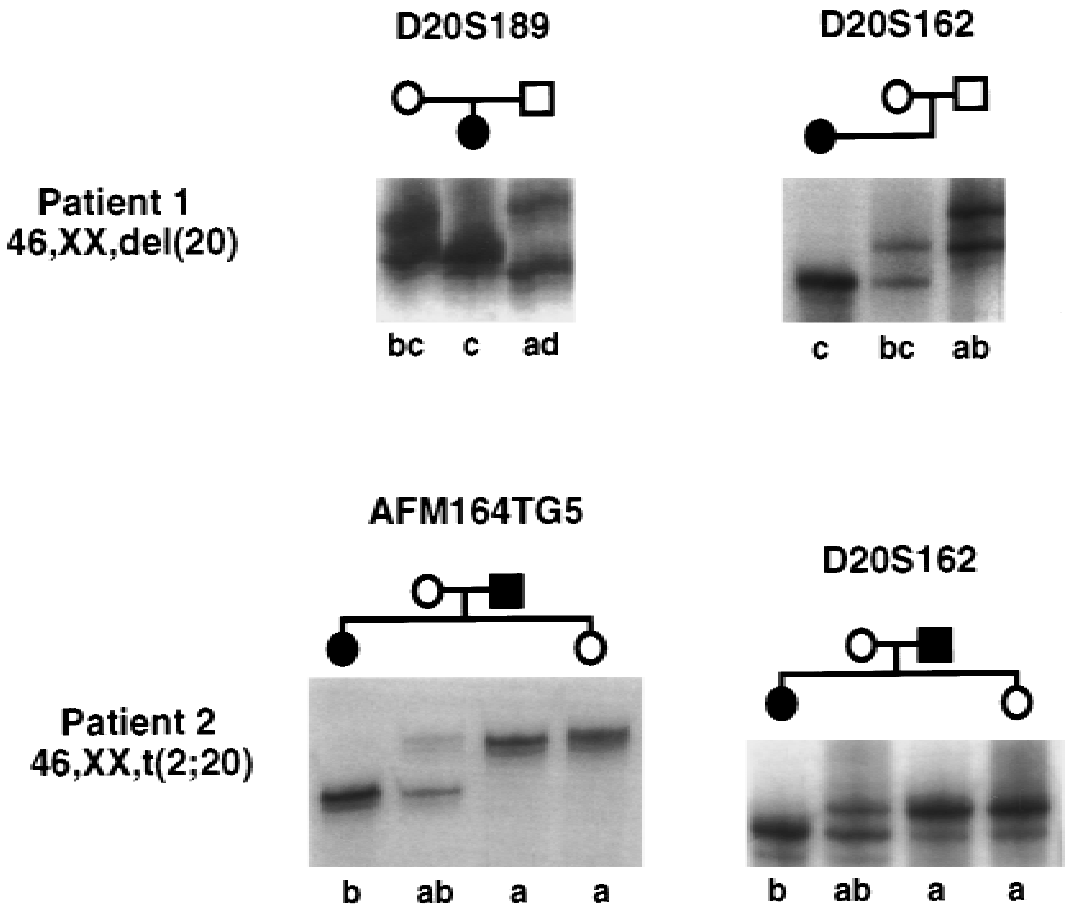


Fig. 2. Analysis of inheritance of chromosome 20 polymorphic loci in Alagille patients with deletions of chromosome 20. Patient 1 [46,XX,del(20)(p11.23p12)] lacks a paternal allele for D20S189 and D20S162. Patient 2 has a reciprocal translocation ([46,XX,t(2;20)(q21.3;p12)], which she has inherited from her affected father. They both have only a single allele at loci AFM164TG5 and D20S162, consistent with a deletion on their shared translocation chromosome.

lateral Axenfeld anomaly. A transhepatic cholangiogram failed to visualize the biliary tree. A chest radiograph showed a "butterfly" vertebra at T-11. Renal ultrasonography documented small echogenic kidneys consistent with parenchymal renal disease. The patient failed to thrive and at age 2 1/2 months a liver biopsy was performed. Although a normal bile duct to portal triad ratio of 1:1 was observed, the biopsy showed cholestasis, portal fibrosis, and epithelial degeneration of bile ducts with focal inflammation suggesting active injury of the bile ducts. (It was observed previously that the number of interlobular bile ducts decreases with age and patients have been reported with cholestasis, inflammation, and a normal portal triad ratio who went on to have bile duct paucity later in childhood [Dahms et al., 1982; Levin et al., 1980].) The patient died at age 19 months of multiple organ failure. An autopsy was not conducted.

A second patient (patient 2) had a reciprocal translocation involving chromosomes 2 and 20 [46,XX,t(2;20)(q21.3p12)], which was also seen in her mildly affected father and sister. This patient was described previously [Spinner et al., 1994] and her clinical findings are summarized in Table II.

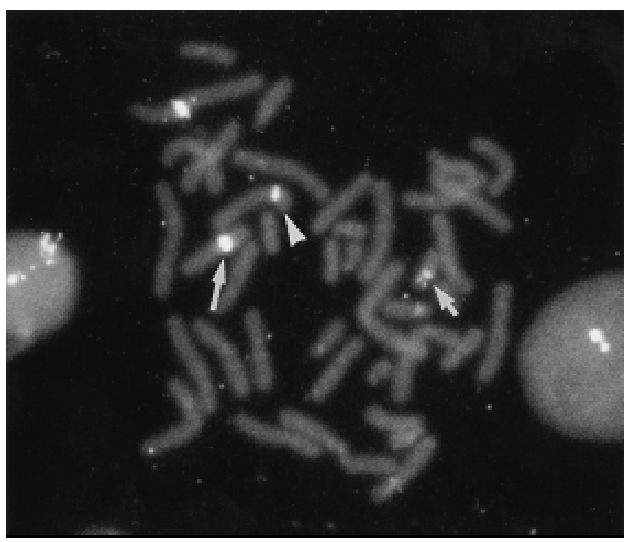

Fig. 3. Fluorescence in situ hybridization of probe D20S5 on a metaphase spread from patient 2, with a t(2;20)(q21.3p12). The centromeres of chromosomes 2 and 20 are marked with an alpha satellite probe. D20S5 is clearly seen on the normal 20 (small arrow) but is absent from the der(2) (long arrow) and the der(20) (arrowhead).

All other patients had apparently normal chromosomes at the 500-850 band level.

### Molecular Deletion Analysis

Patients 1 (46,XX,del(20)) and 2 (46,XX,t(2;20)) were studied at the molecular level in order to further define the cytogenetically identified rearrangements of chromosome 20. Nine polymorphic markers from 20p12 were studied (see Table I for the order of loci in this region). Patient 1 (46,XX,del(20)) was heterozygous (not deleted) for the proximal loci D20S61 and D20S41, uninformative for D20S186, and deleted for D20S189 and D20S162 (Table I, Fig. 2). Intervening markers D20S27, D20S188, and AFM164TG5 were uninformative but are presumably deleted (Table I).

Patient 2 has a deletion in the vicinity of the translocation breakpoint on chromosome 20. Our previous studies using a somatic cell hybrid established from this patient had shown that loci D20S61 and D20S41 were present in a hybrid containing the der(20) and therefore were proximal to the translocation breakpoint [Spinner et al., 1994]. Further analysis of the inheritance of additional polymorphic markers demonstrates that D20S186, D20S189, and D20S27 are uninformative in this family. However, markers AFM164TG5 and D20S162 are deleted in the proposita and her father, both of whom are carriers of the translocation (Table I, Fig. 2). In addition, FISH analysis demonstrated a deletion of D20S5 from the translocated chromosomes (Fig. 3).

Patient 3 (46,XX) has a submicroscopic deletion that was reported previously [Rand et al., 1995]. Deleted loci include D20S61, D20S41, D20S186, and D20S188 and presumably intervening markers D20S189 and D20S27 (Table I).

Comparison of the loci involved in the deletions in these three patients shows that D20S186, D20S189, D20S27, D20S188, and AFM164TG5 define the smallest commonly deleted region (Table I). This region is proximally flanked by D20S41 as it is heterozygous in both patients 1 and 2 and distally by D20S162 as it is heterozygous in patient 3.

Molecular analysis was carried out on 19 patients with normal karyotypes in addition to those reported previously [Rand et al., 1995], using from five to ten markers within and flanking the Alagille syndrome critical region (D20S61, D20S41, D20S186, D20S189, D20S27, D20S188, AFM164TG5, D20S162, and D20S175). None of the patients studied demonstrated a deletion of these markers. However, five of these patients were uninformative for at least three consecutive markers within the commonly deleted region (data not shown). Further studies are in progress to rule out deletions in these patients.

### Clinical Findings

Table II summarizes the manifestations of the Alagille syndrome patients studied. All patients with deletions demonstrated abnormalities in five of five systems affected in Alagille syndrome (liver, heart, skeleton, eye, and facial characteristics). In addition, one of the three patients had renal abnormalities.

Of the 53 patients without demonstrable deletions, 11 were studied by cytogenetics alone, and 42 were studied by molecular and cytogenetic techniques. These patients are listed separately as the possibility of submicroscopic deletions has not been eliminated in the patients studied by cytogenetics alone; 37 of the 53 patients had clinical investigations of all five systems and 14 of the 37 (38%) had abnormalities in every system. All 53 patients in the non-deleted group had cardiac abnormalities (100%), and 52 of 53 (98%) of the patients had hepatic disease. The single exception was a child with heart disease and facial anomalies whose father had Alagille syndrome, including hepatic, cardiac, facial, and renal manifestations. Forty-four of the index cases had ophthalmologic investigations and of these thirty-seven (84%) had posterior embryotoxon or other abnormalities. Fifty had skeletal studies and of these twenty-six (52%) had "butterfly" vertebrae or other skeletal abnormalities. A characteristic face was diagnosed in 43 of 46 (93.4%) patients evaluated.

## CONCLUSIONS

Prior to our studies, multiple cases of Alagille syndrome associated with deletions of the short arm of chromosome 20 (bands 20p11.23-p12) had been reported (Table II). We studied a series of Alagille patients by molecular and cytogenetic techniques to determine the frequency of alterations of this region of the genome. Two of fifty-six patients studied by cytogenetic analysis demonstrated abnormalities involving 20p11.23-p12. A total of 45 patients was studied by molecular methods (24 previously reported [Rand et al., 1995] and 21 presented in this study), and of these three had molecularly detectable deletions of 20p. These included one patient with a submicroscopic deletion of 20p [Rand et al., 1995], and the two patients with cytogenetically detectable rearrangements. We have shown that the reciprocal translocation seen in one patient has an associated deletion of 20p12. Combined molecular and cytogenetic analyses demonstrated that 6.7% (3 of 45 patients) have deletions of 20p11.23-p12.

The finding of multiple patients with 20p deletions led to the hypothesis that Alagille syndrome is a contiguous gene deletion syndrome [Schnittger et al., 1989]. In the Prader-Willi syndrome, 50–60% of patients have a cytogenetically visible deletion of 15q11 [Butler et al., 1986] and another 10% have deletions detectable only by molecular or molecular cytogenetic means [Knoll et al., 1993]. In velo-cardio-facial syndrome, 20% of patients have a visible deletion of 22q11 and over 80% have deletions of the same region detectable at the molecular level [Driscoll et al., 1992]. The frequency of deletions in Alagille syndrome is significantly lower than that seen in other syndromes associated with deletions that are hypothesized to be contiguous gene deletion disorders [Zhang et al., 1990; Deleuze et al., 1994; Rand et al., 1995]. The fact that so few patients with Alagille syndrome have been found to have a deletion argues in favor of a single gene as the cause of this disorder. Other syndromes associated with cytogenetic deletions have been shown to be

TABLE II. Manifestations of Patients Studied and Patients With Deletions of 20p Encompassing the Alagille Syndrome Critical Region*

| | | Sex | Age | Hepatic | Cardiac | Ophtho | Vertebral | Facies | Devel. delay | Renal | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patients from this series | | | | | | | | | | | |
| Patient (1) | 46,XX,del(20)(p11.23p12) | F | 19 mo | + | + | + | + | + | − | + | |
| Non-deleted (N = 53) | 46,XX/XY | | | | | | | | | | |
| | cyto only | 6F/5M | — | 11/11 | 11/11 | 9/11 | 6/11 | 11/11 | 2/11 | 3/11 | |
| | cyto and molec | 20F/22M | — | 41/42 | 42/42 | 28/33 | 20/39 | 32/35 | 0/29 | 8/42 | Retinitis pigmentosa (1 pt) |
| Spinner et al., 1994 (pt. 2 this report) | 46,XX,t(2:20)(q21-p12)[a] | F | 15 yr | + | + | + | + | + | − | − | |
| Rand et al., 1995 (pt. 3 this report) | 46,XX[a] | F | 5 yr | + | + | + | + | + | − | − | |
| Patients reported as having Alagille syndrome | | | | | | | | | | | |
| Byrne et al., 1986 | 46,XX,del(20)(p11.2-pter) | F | 15 mo | + | + | NR | + | + | + | + | Bowel atresia/stenosis digital abnormalities |
| Schnittger et al., 1989 | 46,XX,del(20)(p11.22-p12.2) | F | 20 yr | + | + | + | + | + | + | − | Hearing loss vaginal/uterine agenesis |
| Zhang et al., 1990 | 46,XY,del(20)(p11.2-p12.3) | M | 11 yr | + | + | + | + | − | NR | NR | |
| Legius et al., 1990 | 46,XY,del(20)(p11.2-pter) | M | 5 mo | + | + | − | + | + | + | − | Cleft plate |
| Anad et al., 1990 case 1 | 46,XX,del(20)(p11.23-p13) | F | 13 yr | − | + | + | + | + | + | + | Hearing loss, digital abnormalities |
| Anad et al., 1990 case 2 | 46,XX,del(20)(p12-p13) | F | 4 yr | + | + | − | + | + | + | − | Hearing loss, digital abnormalities |
| Anad et al., 1990 case 3 | 46,XX,del(20)(p11.2-p12) | F | 14 mo | + | + | + | − | + | − | − | |
| Anad et al., 1990 case 4 | 46,XY,del(20)(p11.23-p12.2) | M | 3 yr | + | + | + | − | + | + | + | |
| Anad et al., 1990 case 5 | 46,XX,del(20)(p11.23-p12.2) | F | 30 yr | − | + | + | NR | + | + | NR | |
| Teebi et al., 1992 | 46,XY,del(20)(p11.2-pter) | M | 8 mo | + | + | + | − | + | + | + | |
| Patients not reported as having Alagille syndrome | | | | | | | | | | | |
| Kalousek and Therien 1976 | 46,XX,del(20)(p11-pter) | F | 11 mo | NR | + | NR | + | + | + | − | Bowel atresia/stenosis |
| Kogame et al., 1978 | 46,XY,del(20)(p11-pter) | M | 5 yr | + | + | NR | + | + | + | − | Hearing loss, digital abnormalities, spina bifida |
| Garcia-Cruz et al., 1985 | 46,XY,del(20)(p12.2-pter) | M | 16 yr | − | + | − | + | + | + | − | Hearing loss |
| Vianna-Morgante et al., 1987 | 46,XY,del(20)(p11.2-pter) | M | 14 mo | − | − | NR | NR | + | + | − | Digital abnormalities scoliosis, ear pits |
| Silengo et al., 1988 | 46,XX,del(20)(p11)/46,XX | F | 10 mo | NR | + | NR | + | + | + | + | Double collecting system |

TABLE II. (*Continued.*)

| | | Sex | Age | Hepatic | Cardiac | Ophtho | Vertebral | Facies | Devel. delay | Renal | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kiss and Osztovics, 1988 | 46,XX,del(20) (p11-pter) | F | 6 mo | NR | + | NR | NR | + | + | – | Cleft palate |
| Shohat et al., 1991 | 46,XX,del(20) (p11.23-pter) | F | 6.5 yr | NR | + | + | NR | + | + | NR | Rieger eye anomaly hearing loss |
| Dutta et al., 1991 | 46,XX,del(20) (p11) | F | 6.5 yr | – | – | NR | – | + | + | – | Skeletal abnormalities including spina bifida palpebral dermoid |

*+, finding present; –, finding absent; NR, not reported; M, male; F, female.
[a]Deletions detected by molecular analysis only.

caused by abnormalities of a single gene. Rubinstein-Taybi syndrome (characterized by mental retardation, facial abnormalities, and broad thumbs and toes) is associated with deletions of 16p in 25% of patients. Recent work has demonstrated that mutation of a single gene (CREB Binding Protein) mapping within the critical region causes the complete phenotype of the disorder [Petrij et al., 1995]. It is also still possible that Alagille syndrome is due to loss or disruption of multiple genes, but the distribution of markers within this region is such that we are currently unable to detect small deletions that may encompass multiple genes.

Analysis of the three deletions of 20p has contributed to the delineation of a critical region for Alagille syndrome. Previous studies have placed the Alagille syndrome critical region between D20S61 proximally and D20S162 distally [Rand et al., 1995; Schnittger et al., 1989; Spinner et al., 1994; Zhang et al., 1990]. We have now shown that D20S41 (which is immediately distal to D20S61, see Table I) is outside of the critical region, as it is not deleted in two of the patients we have studied with deletions of 20p. Comparison of the deletions in three patients demonstrates that five loci constitute the commonly deleted region (D20S186, D20S189, D20S27, D20S188, and AFM164TG5). Each of these loci is uninformative in at least two of the three patients with chromosome 20p deletions that we have studied. Narrowing of the commonly deleted region will require additional polymorphic markers or alternative probes at the uninformative loci.

Based on the physical map in this region of the genome [Pollet et al., 1995], we estimate that the Alagille syndrome critical region (between loci D20S41 and D20S162) is approximately 1.5 Mb. Using 30 kb as the average gene size, this region could contain as many as 50 genes [Fields et al., 1994]. However, 20p12 is Giemsa positive and presumably a gene-poor region of the genome. Based on the distribution of CpG islands in this region, it has been estimated that there are fewer than ten genes within the critical region. Two candidate genes that fall within this region have been described (Snap and Plcb4). Plcb4, the human homologue of the Drosophila *norpA* gene, has been shown to block invertebrate phototransduction and lead to retinal degeneration, and was recently mapped to 20p12 [Alvarez et al., 1995]. This gene presents as an attractive candidate gene since retinal degeneration is a component of Alagille syndrome. Preliminary studies indicate that this gene maps distal to the critical region [Krantz et al., 1996]. Snap was mapped within the previously defined Alagille syndrome critical region [Pollet et al. 1995] and work in our laboratory is currently in progress to position it relative to the deletions in our patients [Krantz et al., 1996].

The phenotypes of patients reported with deletions of 20p11-12 are presented in Table II. Ten of 18 patients were reported as having Alagille syndrome. The remaining patients each had some signs of the syndrome, although not all affected systems were investigated. Aside from many of the typical anomalies of Alagille syndrome, six of these 18 patients (33%) demonstrated renal abnormalities. This number is not apparently different than the percentage of Alagille patients without deletions who demonstrate renal anomalies (11 of 53 or 21% in our series). Four of the reported patients with cytogenetic deletions of 20p had hearing loss. This has not been observed in Alagille patients without deletions. Most deleted patients (17 of 20) also demonstrated developmental delay. Developmental delay is not consistently seen in Alagille syndrome. Sixteen percent of 80 patients reported by Alagille et al. [1987] were mentally retarded and in our series only 3 of 56 patients had convincing evidence of delay. However, developmental delay is difficult to evaluate in a chronically ill child who is nutritionally debilitated. The higher frequency of developmental delay in patients with cytogenetically visible deletions of 20p may reflect the large number of genes involved in the deletions. Neither of the two patients we identified with submicroscopic (and presumably smaller) deletions [patient 2 (46,XX,t(2;20) and patient 3 (46,XX)] demonstrated developmental delay. The patient reported here with a larger deletion was developmentally delayed, although her severe clinical course may have contributed.

The hypothesis that there is a small number of genes within the Alagille syndrome critical region is supported by our finding that the phenotype of individuals with visible deletions of 20p12 is not appreciably different from the phenotype of non-deleted patients

(Table II). This, and our finding of a low percentage (~6.5%) of deletions in Alagille syndrome patients, is consistent with this disorder resulting from abnormalities of a single gene.

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